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(54) Title: METHODS FOR GENE TRANSFER USING PSEUDOTYPED LENTIVIRUSES

(57) Abstract: Methods for introducing nucleic acid sequences into hepatocytes, brain glial cells and airway epithelial cell are provided. The methods use filoviral and togaviral glycoprotein-pseudotyped lentiviruses. The viruses comprise a lentiviral capsid and a viral envelope further comprising a lipid bilayer and a functional filoviral glycoprotein or two functional togaviral glycoproteins. In one embodiment the lentivirus is a feline immunodeficiency virus (FIV). In an alternate embodiment, the filoviral glycoprotein is a Marburg virus glycoprotein. In another embodiment, the Marburg virus glycoprotein can have a mutation in the C-terminal portion of the amino acid sequence that results in a higher titer production of the pseudotyped virus. In an alternate embodiment the togaviral glycoproteins are alphavirus glycoproteins, for example, the E1 and the E2 envelope glycoproteins of Ross River virus (RRV).

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METHODS FOR GENE TRANSFER USING PSEUDOTYPED LENTIVIRUSES

FIELD OF THE INVENTION

This invention relates generally to methods for gene transfer to cells using pseudotyped lentiviruses and more specifically to methods for gene transfer using togaviral and filoviral glycoprotein-pseudotyped lentiviruses.

BACKGROUND OF THE INVENTION

Gene therapy is one of the fastest growing areas in experimental medicine. However most studies are only Phase I or Phase II clinical studies designed mainly to evaluate the toxicity of the viral vectors and constructs being used. A major drawback has been the design of vectors that are both safe and efficacious.

Recently retroviruses have generated a great deal of interest for use as viral vectors. One major drawback for retroviral vectors designed to date is their inability to transduce non-dividing cells, such as airway epithelium, hepatocytes and brain glial cells. Retroviral vectors used in *ex vivo* and *in vivo* transduction of hepatocytes required inducing the hepatocytes to proliferate by complex and artificial procedures. One clinical trial was conducted to treat familial hypercholesterolemia by retroviral-mediated *ex vivo* gene transfer. The LDL receptor gene was introduced into hepatocytes that had been surgically removed from patients and which were then reinfused into the liver following gene transduction. There was no convincing evidence, however, of therapeutic efficacy. Liver biopsies were removed after treatment, and few cells tested positive for the expression of LDL-receptor, indicating that transduction efficiency was not high. *In vivo* retroviral-mediated transduction of hepatocytes was even more complicated, as it required artificial regeneration of the liver to give dividing cells. Ferry, N. et al., *Hum. Gene Ther.* 9:1975 (1998).

Retroviral vectors offer several potential advantages for attaining persistent expression of a therapeutic gene in airway epithelium for diseases such as cystic fibrosis. However, several problems have limited their application.

The airway epithelium possesses several unique properties that make it a formidable target for successful gene transfer. Among these are the many innate and adaptive host defense functions that the epithelium and resident immune effector cells perform. The pulmonary epithelium has evolved to prevent the invasion of the host

by microbes and these same strategies may act as barriers for gene transfer vectors. Advances in the field of gene transfer to airway epithelial cells have occurred as an understanding of the cell biology of epithelial host defenses and virus-cell interactions has increased.

Recombinant vectors based on Moloney murine leukemia virus (MoMLV) were the first retroviruses used for gene transfer to airway epithelium. Several studies demonstrated the potential for MoMLV-based vectors to persistently transduce airway epithelium by showing that the retroviral vectors could transduce airway epithelium *ex vivo* and furthermore, that the Cl-transport defect in cystic fibrosis (CF) airway epithelial cells was corrected by transducing the cells *in vitro* with a MoMLV retrovirus vector expressing the CFTR cDNA. However, MoMLV-based vectors require cell division in order for the integration complex to enter the nucleus. However, the normal airway epithelium is mitotically quiescent with less than 1% of the cells dividing. Therefore, transduction efficiency is low in airway epithelial cells.

Thus it would be desirable to have a retroviral vector that can efficiently transduce non-dividing cells, particularly hepatocytes, brain glial cells airway epithelial cells. It would be further desirable if such vectors were efficient in transducing hepatocytes, brain glial, and airway epithelial cells *in vivo*.

SUMMARY OF THE INVENTION

Provided in the present invention are methods for introducing nucleic acid sequences encoding a desired protein into a hepatocyte, brain glial or airway epithelial cell using a filoviral or togaviral glycoprotein pseudotyped lentiviruses. In one embodiment, the viruses can be used *in vitro* to introduce a nucleic acid sequence into a cell. In another embodiment, the viruses of the present invention are used for *in vivo* introduction of a nucleic acid sequence into a hepatocyte, brain glial or airway epithelial cell. In yet another embodiment, the nucleic acid sequence encodes CFTR and the cells are airway epithelial cells and the methods further comprise application of the pseudotyped virus to the apical surface of the airway. In an alternate embodiment the nucleic acid sequence encodes for the LDL receptor, alpha1-antitrypsin, ornithine transcarbamylase, Factor VIII or a high affinity glutamate transporter. The methods further comprise application of the pseudotyped lentivirus to the liver and the brain.

Filoviral and togaviral glycoprotein-pseudotyped lentiviruses are provided for use in the methods of the present invention. The viruses comprise a lentiviral capsid and a viral envelope further comprising a lipid bilayer and a functional filoviral glycoprotein or two functional togaviral glycoproteins. In one embodiment the lentivirus is a feline immunodeficiency virus (FIV). In an alternate embodiment the filoviral glycoprotein is a Marburg or Ebola virus glycoprotein. In a further embodiment the Marburg virus glycoprotein has a mutation in the C-terminal portion of the amino acid sequence that results in a higher titer production of the pseudotyped virus. The Marburg virus glycoprotein can have a C671A or a Y679 stop mutation. Pseudotyped virus comprising Marburg virus glycoprotein with at least one of these mutations have at least a two-fold increase in virus titer production. In an alternate embodiment, the togaviral glycoproteins are alphavirus glycoproteins, preferably the E1 and E2 envelope glycoproteins of Ross River virus (RRV).

Additional objects, advantages, and features of the present invention will become apparent from the following description, taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE FIGURES

The various advantages of the present invention will become apparent to one skilled in the art by reading the following specification and by referencing the following drawings in which:

Figure 1A is a photograph of the en face view of duplicate samples of x-gal stained liver slices showing the efficiency of transducing hepatocytes with RRV pseudotyped FIV having a β-galactosidase reporter gene;

Figure 1B is a photograph of the en face view of duplicate samples of x-gal stained liver slices showing the efficiency of transducing hepatocytes with VSV-G pseudotyped FIV having a β-galactosidase reporter gene;

Figure 1C is a photograph of the en face view of duplicate control samples of x-gal stained liver slices treated with PBS;

Figure 1D is a photograph of a liver slice stained with hematoxylin and eosin showing efficiency of transducing hepatocytes with RRV pseudotyped FIV having a β-galactosidase reporter gene;

Figure 1E is a photograph of a liver slice stained with hematoxylin and eosin showing efficiency of transducing hepatocytes with VSV-G pseudotyped FIV having a β-galactosidase reporter gene;

Figure 1F is a photograph of a control liver slice stained with hematoxylin and eosin which was treated with PBS;

Figure 2A is a bar graph showing the effect of RRV pseudotyped FIV and VSV-G pseudotyped FIV on liver function as measured by serum SGPT levels;

Figure 2B is a bar graph showing the effect of RRV pseudotyped FIV and VSV-G pseudotyped FIV on liver function as measured by serum SGOT levels;

Figure 3A is a photograph of showing the production of β-galactosidase by astrocytes transduced with RRV-pseudotyped FIV ;

Figure 3B is a photograph of showing the production of GFAP by astrocytes transduced with RRV-pseudotyped FIV ;

Figure 3C is a photograph of showing the production of β-galactosidase and GFAP by astrocytes transduced with RRV-pseudotyped FIV ;

Figure 4A is a photograph showing the production of β-galactosidase by oligodendrocytes transduced with RRV-pseudotyped FIV;

Figure 4B is a photograph showing the production of CNPase by oligodendrocytes transduced with RRV-pseudotyped FIV;

Figure 4C is a photograph showing the production of β-galactosidase and CNPase by oligodendrocytes transduced with RRV-pseudotyped FIV;

Figure 5A is a photograph showing the production of β-galactosidase by oligodendrocytes transduced with RRV-pseudotyped FIV;

Figure 5B is a photograph showing the production of CNPase by oligodendrocytes transduced with RRV-pseudotyped FIV;

Figure 5C is a photograph showing the production of β-galactosidase and CNPase by oligodendrocytes transduced with RRV-pseudotyped FIV;

Figure 6 is a table showing the selective transduction of CNS cell types by FIV vectors pseudotyped with RRV envelope glycoproteins.

Figure 7A is a photograph showing gene transfer in human airway epithelia that were exposed on the apical surface to FIV pseudotyped with VSV-G;

Figure 7B is a photograph showing gene transfer in human airway epithelia that were exposed on the basolateral surface to FIV pseudotyped with VSV-G;

Figure 7C is a photograph showing gene transfer in human airway epithelia that were exposed on the apical surface to FIV pseudotyped with Marburg glycoprotein;

Figure 7D is a photograph showing gene transfer in human airway epithelia that were exposed on the basolateral surface to FIV pseudotyped with Marburg glycoprotein;

Figure 8 is a schematic showing mutations at the C-terminus of the amino acid sequences of the Marburg envelope glycoprotein (SEQ. ID. NOs: 1-7);

Figure 9 is a bar graph showing the effect of mutations at the C-terminus of the Marburg envelope glycoprotein on the titer of FIV pseudotyped with the mutant Marburg glycoproteins; and

Figure 10 is a schematic showing the amino acid sequence (SEQ. ID. NO: 8) of the Marburg envelope glycoprotein.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods for introducing nucleic acid sequences encoding a desired protein into a hepatocyte, brain glial or airway epithelial cell using a filoviral or togaviral glycoprotein pseudotyped lentivirus. In one embodiment, the viruses can be used *in vitro* to introduce a nucleic acid sequence into a cell. In another embodiment, the viruses of the present invention are used for *in vivo* introduction of a nucleic acid sequence into a hepatocyte, brain glial or airway epithelial cell. In yet another embodiment, the nucleic acid sequence encodes CFTR and the cells are airway epithelial cells and the methods further comprise application of the pseudotyped virus to the apical surface of the airway. In an alternate embodiment the nucleic acid sequence encodes for the LDL receptor, alpha1-antitrypsin, ornithine transcarbamylase, Factor VIII or a high affinity glutamate transporter. The methods further comprise application of the pseudotyped lentivirus to the liver and the brain.

Filoviral glycoprotein-pseudotyped lentiviruses are provided for use in the methods of the present invention. The viruses comprise a lentiviral capsid and a viral envelope further comprising a lipid bilayer and a functional filoviral glycoprotein. In

one embodiment the lentivirus is a feline immunodeficiency virus (FIV). In an alternate embodiment the viral glycoprotein is a Marburg virus glycoprotein. The Marburg virus glycoprotein can have a mutation in the C-terminal portion of the amino acid sequence that results in a higher titer production of the pseudotyped virus. In a further embodiment, the Marburg virus glycoprotein has a C671A or a Y679 stop mutation. Pseudotyped viruses comprising Marburg virus glycoprotein with at least one of these mutations have at least a two-fold increase in virus titer production. In yet another alternate embodiment, the glycoprotein is an Ebola virus glycoprotein.

It will be appreciated by those skilled in the art that conservative substitutions of amino acids can be made without substantially changing the activity or structure of a protein. In one embodiment, the cysteine at position 671 of the Marburg glycoprotein is replaced by an alanine, valine, glycine, isoleucine, or leucine (Figure 8, SEQ. ID.NO: 4). It has been shown that when the cysteine at position 671 is replaced by an aliphatic, non-polar amino acid, the titer of FIV pseudotyped with the mutant Marburg glycoprotein increases about at least 3-fold (Figure 9). In an alternate embodiment, the amino acid sequence of the Marburg glycoprotein is truncated at the C-terminus. In another embodiment, the amino acid sequence is truncated from about isoleucine 680 (I680Stop) to about phenylalanine 676 (F676Stop) (Figure 8, SEQ. ID. NO: 7)). Truncating the amino acid sequence of the C-terminus of the Marburg glycoprotein results in at least a 2-fold increase in the titer of FIV pseudotyped with the truncated glycoprotein (Figure 9).

Togaviral glycoprotein-pseudotyped lentiviruses are also provided for use in the methods of the present invention. The viruses comprise a lentiviral capsid and a viral envelope further comprising a lipid bilayer and two functional togaviral glycoproteins. In one embodiment the lentivirus is a feline immunodeficiency virus (FIV) which has two togaviral glycoproteins imbedded into the lipid bilayer surrounding the capsid. Examples of, but not limited to, togaviral glycoproteins are alphavirus glycoproteins, preferably the E1 and E2 envelope glycoproteins of Ross River virus (RRV). It was recently reported that by manipulating the E1 and E2 RRV glycoproteins so that they were expressed by individual genes in a packaging cell system, a stable cell line producing an RRV-pseudotyped Moloney murine virus was obtained. Sharkey, C.M.. et al., *J. Virol.* 75:2653 (2001).

The pseudotyped viruses of the present invention may further comprise another nucleic acid sequence that encodes a desired protein. The protein can be such that it provides a beneficial or therapeutic effect if introduced into an animal. For example, a gene may encode a protein that is needed by an animal, either because the protein is no longer produced, is produced in insufficient quantities to be effective in performing its function, or is mutated such that it either no longer functions or is only partially active for its intended function. The nucleic acid sequence may be introduced into the pseudotyped virus in a variety of ways known to the skilled artisan. In one embodiment, the nucleic acid sequence encodes for CFTR (cystic fibrosis transmembrane regulator protein), the chloride transporter that is involved in cystic fibrosis. The absence of CFTR function in lung epithelium due to mutations in the gene encoding CFTR, results in a severe lung disease that cannot be readily reversed or controlled by conventional treatment. Lack of CFTR function in the lung results in airway fluid with an altered ion composition, thereby creating a favorable environment for disease-causing bacteria to colonize the lung. Additionally, mucus secreted into the lung becomes thick and viscous, preventing normal clearing of the bacteria from the airways. The chronic bacterial infection leads to destruction of lung tissue and loss of lung function. Replacing the defective gene with a copy that encodes for a functional CFTR can abate the symptoms. In an alternate embodiment, the nucleic acid sequence encodes for the LDL receptor, alpha1-antitrypsin, ornithine transcarbamylase, Factor VIII or a high affinity glutamate transporter. For example, increasing the expression of the LDL receptor in the liver allows for more efficient clearance of LDL-cholesterol from the body.

Alternatively, the desired protein may be one that allows the entry of the virus into a cell to be detected. For example, a visually detectable component, or marker, such as one that emits visible wavelengths of light, or that may be reacted with a substrate to produce color of specified wavelengths. For example, such nucleic acid sequences include the nucleic acid sequence encoding the *Aequorea victoria* green fluorescent protein and the LacZ gene that encodes for beta-galactosidase, both of which are well known in the art and may be obtained commercially.

Methods of introducing a nucleic acid sequence encoding a desired protein into a cell are provided. In one embodiment, the method includes contacting, or transducing, an airway epithelial cell with a lentivirus that has been pseudotyped with

a filoviral glycoprotein that includes the desired nucleic acid sequence in its genome. The level of transduction may be monitored by assaying methods known to the skilled artisan, and include assaying for the protein of interest encoded by the introduced nucleic acid sequences or assaying for the presence of the nucleic acid sequences.

In a surprising result, the pseudotyped viruses of the present invention were found to effectively transduce airway epithelium when introduced to the apical surface of the airways. One of the major barriers to gene therapy in airways is the resistance of airway epithelium to transduction by viral vectors *in vivo*. The apical surface of the epithelial is the surface that the viral vectors contact when provided directly into the airways. Multiple factors present on the apical surface of epithelia may act as physical barriers preventing vector access to receptors. These include mucus, airway surface liquid and its components, immune effector cells, such as macrophages and neutrophils, and the extracellular matrix. Although the viral vectors for transporting CFTR into airway epithelial have been constructed previously, none have been particularly effective.

In another embodiment, the method includes contacting, or transducing, a hepatocyte or brain glial cell with a lentivirus that has been pseudotyped with togaviral glycoproteins that includes the desired nucleotide sequence in its genome. The level of transduction may be monitored by assaying methods known to the skilled artisan, and include assaying for the protein of interest encoded by the introduced nucleotide sequences or assaying for the presence of the nucleotide sequences. In a surprising result, the pseudotyped viruses of the present invention were found to effectively transduce hepatocytes *in vivo*. Previous reports of transduction of hepatocytes by retroviral vectors have suggested that *in vivo* gene therapy for liver defects and diseases would be difficult. Figures 1A-1F show that transduction with RRV-pseudotyped FIV is extensive throughout the liver (Figure 1A), especially when compared to a VSV-G pseudotyped lentivirus control (Figure 1B). Furthermore, the viruses of the present invention do not affect liver function as measured by SGOT and SGPT levels of treated livers (Figures 2A and 2B). This is in stark contrast to the VSV-G pseudovirus (Figures 2A and 2B), which has been reported to be toxic to a variety of cell types.

In an alternate embodiment, the cells are brain glial cells. One type of glial cell, oligodendrocytes, is responsible for formation of the myelin sheath that protects

the spinal cord. In multiple sclerosis, both oligodendrocytes and the myelin sheath are destroyed. Another type of glial cell, astrocytes, contains high affinity glutamate transporters that are critical in maintaining the extracellular glutamate concentration at sub-excitotoxic levels and thereby preventing neuronal cell death. Insufficient glutamate uptake by the transporters is believed to play a role in amyotrophic lateral sclerosis, Alzheimer's disease, schizophrenia, and AIDS by way of non-limiting example. Astrocytic uptake of glutamate may also serve to fine-tune the time course of glutamate in the synaptic cleft, perhaps by terminating the synaptic signal. Additionally, astrocytes may mediate inter-synaptic spillover of glutamate. The togaviral glycoprotein pseudotyped lentiviruses of the present invention are selective for transducing glial cells as compared to other CNS cells. A feline immunodeficiency virus (FIV) pseudotyped with at least two different Ross River (RRV) viral glycoproteins was effective in transducing brain astrocytes (Figures 3A-3C). The presence of the marker protein GFAP confirmed that the brain glial cells were astrocytes. The FIV virus pseudotyped with RRV glycoproteins was also effective in transducing oligodendrocytes (Figures 4A-4C and 5A-5C). The presence of the marker protein CNPase confirmed that the brain glial cells were oligodendrocytes. The data in the table of Figure 6 confirms the selective transduction of astrocytes and oligodendrocytes (oligos) by the togaviral pseudotyped lentivirus as compared to other types of brain cells.

The pseudotyped viruses can be introduced into a mammal requiring gene therapy by a number of ways known to the skilled artisan. For airway epithelium, the viruses can be introduced directly into the airway by inhalation aided by a nebulizer or an inhaler. The pseudotyped lentiviruses of the present invention can also be injected intravenously for systemic gene delivery. The pseudotyped lentiviruses can also be injected directly into the liver or the brain parenchyma. Alternatively, hepatocytes, brain glial cells or airway epithelial cells may be removed from the mammal, transduced with the pseudotyped lentiviruses and then implanted back into the patient.

The present invention also provides methods of screening agents effective in blocking viral entry into a cell. The methods allow for direct screening as the viral entry step can be detected in the method. In one embodiment, the method comprises treating the cell or the virus with the desired agent, contacting the cell with the virus,

and detecting viral entry into the cell. A wide variety of agents may advantageously be screened in the present invention, including, immunological agents such as monoclonal and/or polyclonal antibodies. Alternately, various pharmacological agents may also be screened in the present method in the same way, and may include proteins, peptides and various chemical agents.

In yet another embodiment, kits for forming inventive filovirus glycoprotein-pseudotyped lentivirus are provided. The kits contain the plasmids and nucleic acid sequences required to transform a cell to produce the desired virus.

The foregoing and other aspects of the invention may be better understood in connection with the following example, which is presented for purposes of illustration and not by way of limitation.

EXAMPLE

Methods for preparing and administering pseudotyped vectors to models:

Vector production. The second generation FIV vector system was previously reported. Johnston, J.C. et al, *J Virol.* 73:4991, (1999). Plasmid constructs consist of an FIV packaging construct with a deletion in the *env* gene and mutations in *vif* and *orf2*, an FIV vector construct expressing cytoplasmic *E. coli* β-galactosidase, eGFP or other nucleic acid sequences of interest, and an envelope plasmid in which the human CMV early gene promoter directs transcription of the Marburg envelope cDNA. The FIV packaging plasmid (pCFIVΔ*orf2*Δ*vif*) contains the FIV packaging signal (ψ), the *gag* and *pol* genes, and the *rev* sequences. FIV *rev* is analogous to the HIV *rev* in enabling expression of late genes encoded by unspliced or singly spliced mRNAs containing the cis-acting Rev-responsive element (RRE). The proviral FIV 5' LTR is replaced by the CMV promoter/enhancer and the 3' LTR is replaced with the simian virus 40 polyadenylation signal. A deletion in the *env* gene and mutations in FIV accessory genes *vif* and *orf2* render these sequences inactive without negatively affecting vector titer.

The FIV vector plasmids (based on $pVET_L$) consist of the FIV 5' and 3' LTR sequences flanking a portion of the *gag* sequence including the packaging signal, a transgene cassette, and the RRE. The U3 region of the 5' FIV LTR is replaced with the CMV promoter. A CMV promoter-β-Gal expression plasmid, pCMVβgal, was

generated by combining an *XbaI/SalI* fragment corresponding to the CMV promoter from pCMV-G and a *SalI/SmaI* fragment corresponding to the β-Gal gene from pSP6-β-GAL into pBlueScript SK(−). pTFIVLCβ, pTC/FLCβ, and pTC/FSCβ were then generated by insertion of the *NotI/SmaI* CMV-β-Gal expression cassette from pCMVβ gal into similarly digested pTFIVL, pTC/FL, and pTC/FS vector backbones, respectively. These constructs were renamed pTFIV$_L$Cβ, pVET$_L$Cβ, and pVET$_S$Cβ, respectively. A pCMVβgalCTE expression plasmid was used to generate an FIV expression vector containing the constitutive RNA transport element (CTE) from Mason-Pfizer monkey virus (MPMV). pCMVβgalCTE was constructed in part from pSK-CTE. pSK-CTE was generated by PCR amplification of the CTE with the primers CTEH5 and CTEH3, which harbor *Hin*dIII sites near their 5' ends. The resulting PCR product was digested with *Hin*dIII and inserted into similarly digested pBlueScript SK(−) to generate pSK-CTE. pSK-CTE was then digested with *Sma*I and *Xho*I, and the insert was ligated into similarly digested pCMVβgal to generate pCMVβ galCTE. A *NotI/XhoI* fragment containing the CMVβgalCTE expression cassette from pCMVβgalCTE was then ligated into *NotI/SalI*-digested pTC/FL to create pTC/FLCβ CTE (now referred to as pVET$_L$Cβ$_{CTE}$).

The VSV-G envelope plasmid, pCMV-G, encodes the VSV envelope glycoprotein. Yee, J.K. et al., *Proc. Natl. Acad. Sci. USA* 91: 9564-9568 (1994). The pRRV-E2E1 plasmid encodes the full-length RRV envelpe glycoprotein, E3-E3-6K-E1, which is processed proteolytically into the individual subunits. The region encoding the RRV envelope glycoproteins was amplified from pRR64, which contains the full-length cDNA of the RRV genome (Kuhn, R.H. et al., *Virology* 182: 430-441 (1991)), using *Taq* DNA polymerase (Promega Corporation) and two primers complementary to the viral cDNA at nucleotides 8376 and 11312. The amplified fragment, which contained the RRV E3-E2-6K-E1 coding region, was digested with the restriction endonucleases *Bam*HI and *Xba*I and ligated into the *Bam*HI and *Xba*I sites of pBacPac, a baculovirus expression vector (Clontech). The resulting plasmid was digested with *Bam*HI and *Xba*I, and the fragment containing the RRV E3-E2-6K-E1 coding region was ligated into the *Bam*HI and *Xba*I sites in the pcDNA3 and pcDNA3.1/Zeo(+) mammalian expression vectors (Invitrogen). The resulting plasmids were designated pRRV-E2E1 and pRRV-E2E1A, respectively.

To construct this plasmid, the nucleotides 5931-8033 from the Marburg virus genome (SEQ. ID. NO: 8, Genbank Accession Number Z12132) were cloned into the pSP72 plasmid (from Promega) under the control of the T7 promoter using SalI. The XhoI and Eco RI fragment of this plasmid was cloned into the XhoI and Eco RI polylinker sites of the mammalian expression vector pcDNA3. SEQ ID 9 also shows the amino acid sequence of the Marburg virus glycoprotein

Pseudotyped FIV vector particles were generated by transient transfection of plasmid DNA into 293T cells plated 1 day prior to transfection at a density of 2.8×10^6 per 10-cm-diameter culture dish as described by Johnston, J.C. et al, *J Virol.* 73:4991, (1999). Three plasmid cotransfections were performed using packaging, envelope, and vector plasmids, followed by collection of supernatants and particle concentration by centrifugation. For each preparation, 750 ml of culture supernatant was centrifuged overnight at 7,400 x g and resuspended in 3 ml of lactose buffer (19.5 mM Tris at pH 7.4, 37.5 mM NaCl, and 40 mg/ml lactose). Transduction titers before and after concentration were determined by measurement of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)-positive cells in tranduced HT-1080 target cells and wre expressed as tranducing units (TU)/ml.

Gene transfer methods

Lung

In vitro. To transduce differentiated human epithelia, the pseudotyped FIV vector was mixed with cell culture medium to a final volume of 100 μl (MOI ~10). This mixture was applied to either the apical surface or the basal surface of primary cultures of human airway epithelia as described previously. Wang, G. et al., *J Virol.* 104: R49-R56, (1999). To enhance transduction from the apical surface, vector was mixed at a 1:1 (vol/vol) ratio with 12 mM EGTA HEPES/saline solution (pH 7.3), and applied apically for 4 hours as previously reported for Murine leukemia virus vectors. Wang, G. et al., *J Virol.* 104: R49-R56, (1999). The results are shown in Figure 1. The pseudotyped FIV vector was effective in transducing cells when applied to either the basal or apical surface of the cells. In contrast, the VSV-G control could not transduce the cells when applied to the apical surface.

In vivo. For tracheal gene transfer, adult New Zealand white rabbits are anesthetized with 32 mg/kg ketamine, 5.1 mg/kg xylazine and 0.8 mg/kg

acepromazine intramuscularly, a ventral midline incision made and tracheotomy performed. An approximately 1.5 cm tracheal segment cephalad to the tracheotomy was isolated and cannulated on each end with PE 330 tubing (Clay Adams, Becton Dickinson). The tracheal segment was rinsed and then filled with a FIV-β–gal vector solution. The vector solution was left in place for 45 min, then the cannulae were removed and the incisions closed. Five days or 6 weeks later, the tissues are studied for β-galactosidase expression. For lower airway gene transfer, a PE50 catheter was passed via the trachea until it lodged in a subsegmental bronchus. 200-600 μl of FIV-β–gal of various envelope pseudotypes was instilled. Five days later, the tissues are studied for β-galactosidase expression.

Brain

Six to 8 week old adult male C57BL/6 mice were used for gene transfer. Mice were anaesthetized and 5 x 10^5 TU of the vectors were stereotactically injected into either the right lateral ventricle or the right striatum, using a 26 gauge Hamilton syringe driven by a microinjector (Micro 1, World Precision Instruments, Sarasota, FL) at 0.5 μl per minute. For ventricular injections, 10 μl volumes were injected at coordinates 0.4 mm anterior, 1.0 mm lateral to bregma at 2 mm depth. For striatal injections, 5 μl volumes are injected at coordinates 0.4 mm rostral and 2 mm lateral to bregma, and at a 3 mm depth. A minimum of two independent experiments are done for each vector and injection site. At 3 weeks postinjection, mice were sacrificed and perfused with 2% formaldehyde in PBS. The brains were postfixed overnight at 4 °C and cryoprotected in 30% sucrose-PBS for 48 h at 4 °C. The hemispheres were separated and blocked in O.C.T. (Sakura Finetek USA, Torrance, CA) by freezing in a dry ice-ethanol bath. Parasagittal cryosections (10 μm) were cut and placed on slides. Slides were stained with X-Gal or were dually stained with antibodies for immunofluorescent confocal analysis.

Liver

The C57Bl/6 mice were intravenously injected via tail vein with FIV vector (total dose 1.3x10^7 to 6x10^7 IU), administrated over one or on two consecutive days (one injection/day). Controls received vector buffer. The injection volume was 0.4

ml. On days 1 and 7 postinjection, blood samples were obtained from the retro-orbital plexus and the serum samples assayed for the levels of glutamic oxalacetic transaminase (SGOT) and glutamic pyruvic transaminase (SGPT) using a transaminase assay kit (Sigma, St. Louis, MO) according to the manufacturer's instructions. At 3 weeks postinjection, the mice were sacrificed and perfused with cold phosphate-buffered saline (PBS). Samples of liver, spleen, kidney, lung, heart, and skeletal muscles (triceps) were harvested for X-Gal staining.

Determination of β-galactosidase expression

For X-Gal staining of liver after intravenous vector injection, lobes were fixed in 2% paraformaldehyde-PBS overnight and then stained with X-Gal overnight at 4 °C. The overall expression of b-galactosidase was first examined by stereo microscopy. The X-Gal-stained tissue was then embedded in paraffin, and 5-μm sections were cut at 50-μm intervals and counterstained with hematoxylin and eosin for quantification and histological examination. For X-Gal staining of brain and muscle sections, 10-mm sections on slides were incubated in X-Gal for 6 h at 37 °C, washed in PBS, and counterstained with neutral red. For X-Gal staining of lung, the lungs were removed, inflated with and submersed in 2% paraformaldehyde-PBS, and allowed to fix for 4 h at 4 °C. After fixation, the lungs were washed with PBS and inflated with X-Gal solution. The lungs were submersed in additional X-Gal and incubated overnight at 37 °C. After X-Gal staining, the lungs were washed with PBS and paraffin embedded by a standard protocol, and 10-μm sections were collected. Sections were counterstained with nuclear fast red.

Immunostaining

To determine the cell types transduced after intrastriatal injections of RRV pseudotyped FIV, 10-μm brain sections were dually stained for β-galactosidase and glial fibrillary acidic protein (GFAP, a type II astrocyte-specific intermediate filament), or for β-galactosidase and 2',3'-cyclic nucleotide-3'-phosphodiesterase (CNPase, an oligodendrocyte-myelin specific marker) and analyzed by confocal fluorescence microscopy. The antibodies used were polyclonal rabbit anti-β-galactosidase (Biodesign International, Saco, ME), Cy3-conjugated mouse

monoclonal anti-GFAP (Sigma), mouse monoclonal anti-CNPase (Sigma) Alexa 488-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR) and lissamine-rhodamine-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA). Sections were blocked with 10% normal goat serum and 0.1% Triton X-100 in PBS for 1 h at room temperature and then incubated with primary antibodies overnight at 4 °C in PBS with 3% bovine serum albumin and 0.1% Triton X-100. The sections were then washed, incubated with secondary antibodies for 2 h at room temperature, washed, and coverslipped with gel mount. Using confocal microscipy, images from 0.3-μm-thick Z series were collected.

The foregoing discussion discloses and describes merely exemplary embodiments of the present invention. One skilled in the art will readily recognize from such discussion, and from the accompanying drawings, that various changes, modifications and variations can be made therein without departing from the spirit and scope of the invention.

What is claimed is:

1. A pseudotyped lentivirus comprising:

a lentiviral capsid;

a lipid bilayer wherein said bilayer surrounds said capsid; and

a Marburg glycoprotein disposed in said lipid bilayer wherein the Marburg glycoprotein has the mutation of C671A, F676stop or Y679stop.

2. The lentivirus of claim 1 further comprising a nucleic acid sequence encoding a desired protein, said nucleic acid sequence enclosed within said lentiviral capsid.

3. The lentivirus of claim 2 wherein the desired protein is CFTR.

4. The lentivirus of claim 1 wherein the lentiviral capsid comprises a feline immunodeficiency virus capsid.

5. A method of introducing a nucleic acid sequence encoding a desired protein into an airway epithelial cell comprising the step of transducing an airway epithelial cell with a pseudotyped lentivirus comprising a lentiviral capsid, a lipid bilayer wherein said lipid bilayer surrounds said capsid, a filoviral glycoprotein disposed in said bilayer and a nucleic acid sequence encoding a desired protein.

6. The method of claim 5 wherein said filoviral glycoprotein is a Marburg glycoprotein.

7. The method of claim 6 wherein the Marburg glycoprotein has the mutation of C671A, F676stop or Y679stop.

8. The method of claim 5 wherein the lentiviral capsid comprises a feline immunodeficiency virus capsid.

9. The method of claim 5 wherein the desired protein is CFTR.

10. A method of introducing a nucleotide sequence encoding a desired protein into a hepatocyte or brain glial cell comprising the step of transducing a hepatocyte or brain glial cell with a pseudotyped lentivirus comprising a lentiviral capsid, a lipid bilayer wherein said lipid bilayer surrounds said capsid, at least two different togaviral glycoproteins disposed in said bilayer and a nucleotide sequence encoding a desired protein.

11. The method of claim 10 wherein said togaviral glycoproteins are alphaviral glycoproteins.

12. The method of claim 11 wherein the alphaviral glycoproteins are Ross River alphaviral glycoproteins.

13. The method of claim 10 wherein the retroviral capsid is comprised of a feline immunodeficiency virus capsid.

14. The method of claim 10 wherein the desired protein is the LDL receptor, alpha1-antitrypsin, ornithine transcarbamylase, Factor VIII or a high affinity glutamate transporter.

15. A method for introducing a nucleic acid sequence encoding a desired protein into the airway epithelial cells of a mammal comprising the step of administering to the lungs of the mammal a pseudotyped lentivirus comprising a lentiviral capsid, a lipid bilayer wherein said lipid bilayer surrounds said capsid, a filoviral glycoprotein disposed in said bilayer and a nucleic acid sequence encoding a desired protein wherein the nucleic acid sequence is enclosed within the lentiviral capsid.

16. The method of claim 15 wherein said filovirus glycoprotein is a Marburg glycoprotein.

17. The method of claim 16 wherein the Marburg glycoprotein has the mutation of C671A, F676stop or Y679stop.

18. The method of claim 15 wherein the lentiviral capsid comprises a feline immunodeficiency virus capsid.

19. The method of claim 15 wherein the desired protein is CFTR.

20. A method for introducing a nucleotide sequence encoding a desired protein into the liver or brain of a mammal comprising the step of administering to the mammal a pseudotyped lentivirus comprising a lentiviral capsid, a lipid bilayer wherein said lipid bilayer surrounds said capsid, at least two different togaviral glycoproteins disposed in said bilayer and a nucleotide sequence encoding a desired protein wherein the nucleotide sequence is enclosed within the lentiviral capsid.

21. The method of claim 20 wherein said togaviral glycoproteins are alphaviral glycoproteins.

22. The method of claim 21 wherein the alphaviral glycoproteins are Ross River alphaviral glycoproteins.

23. The method of claim 20 wherein the lentiviral capsid is comprised of a feline immunodeficiency virus capsid.

24. The method of claim 20 wherein the desired protein is the LDL receptor, alpha1-antitrypsin, ornithine transcarbamylase, Factor VIII or a high affinity glutamate transporter.

25. The method of claim 20 wherein the pseudotyped virus is administered to the mammal intravenously.

26. The method of claim 20 wherein the pseudotyped virus is administered by injection directly into the liver or brain.

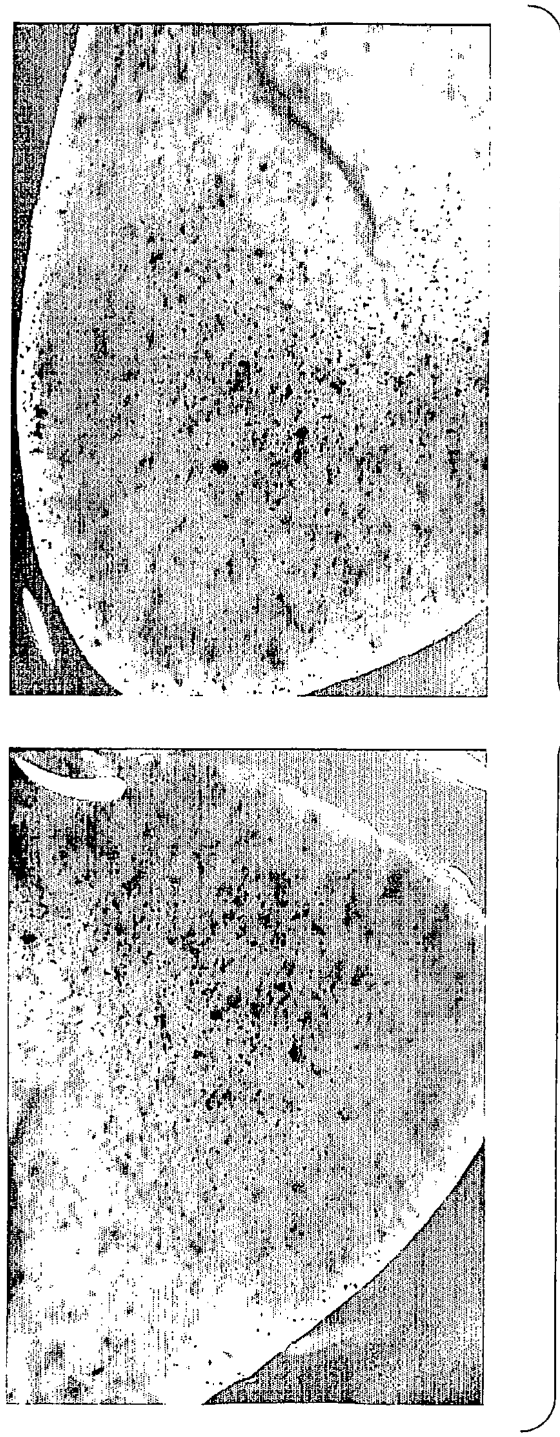


Fig. 1A

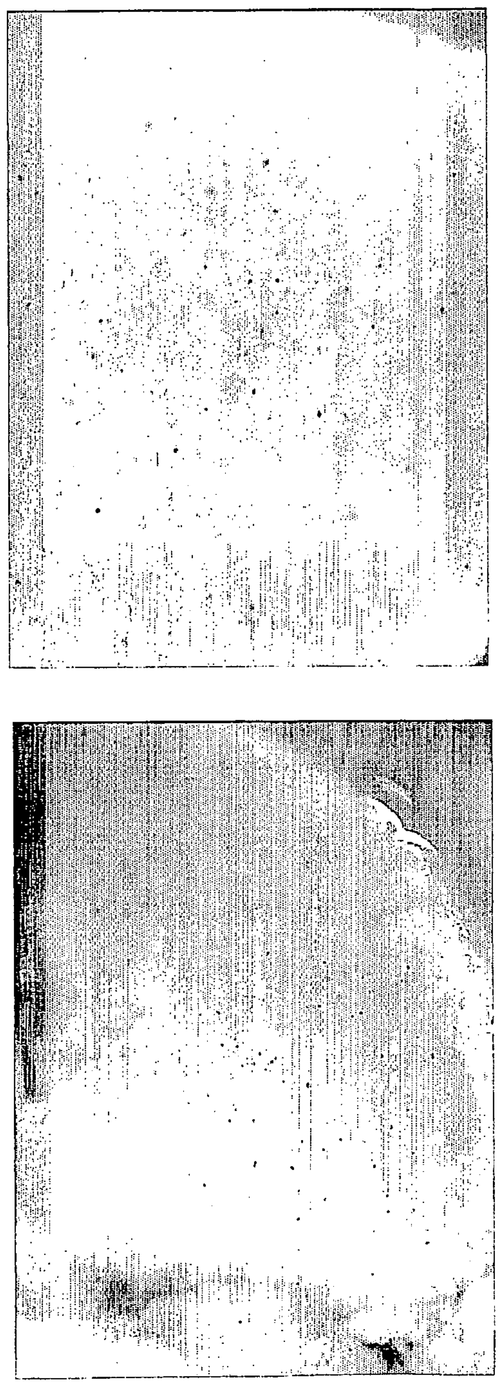


Fig. 1B

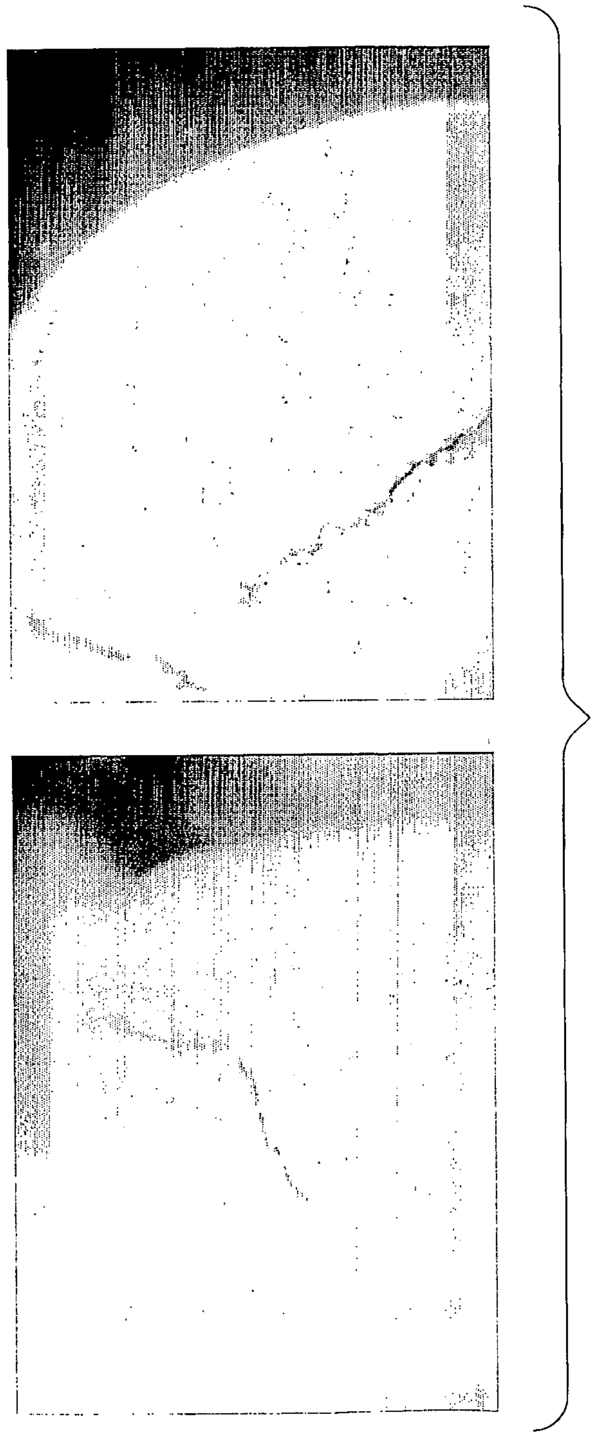
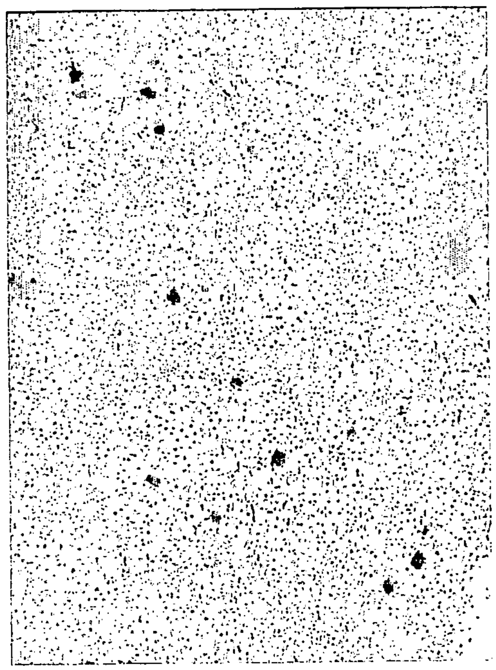


Fig. 1C



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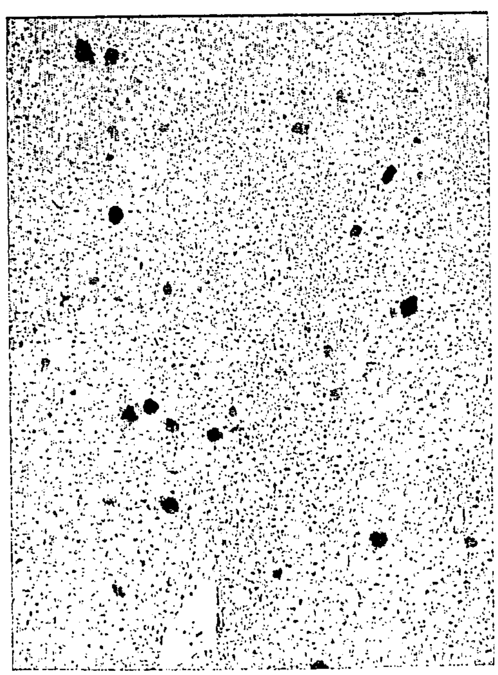

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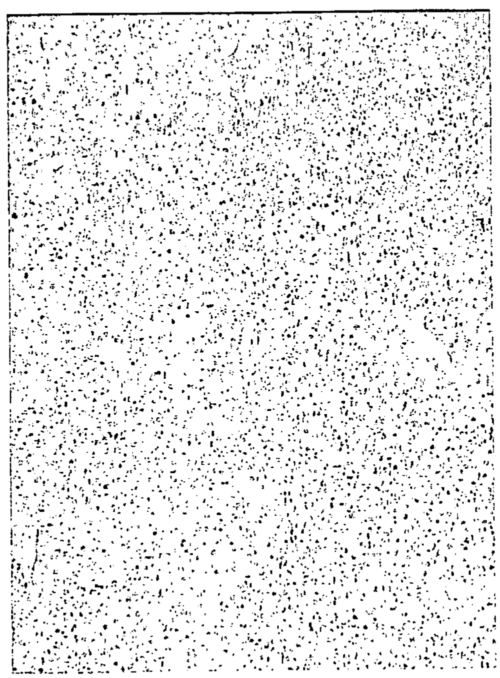

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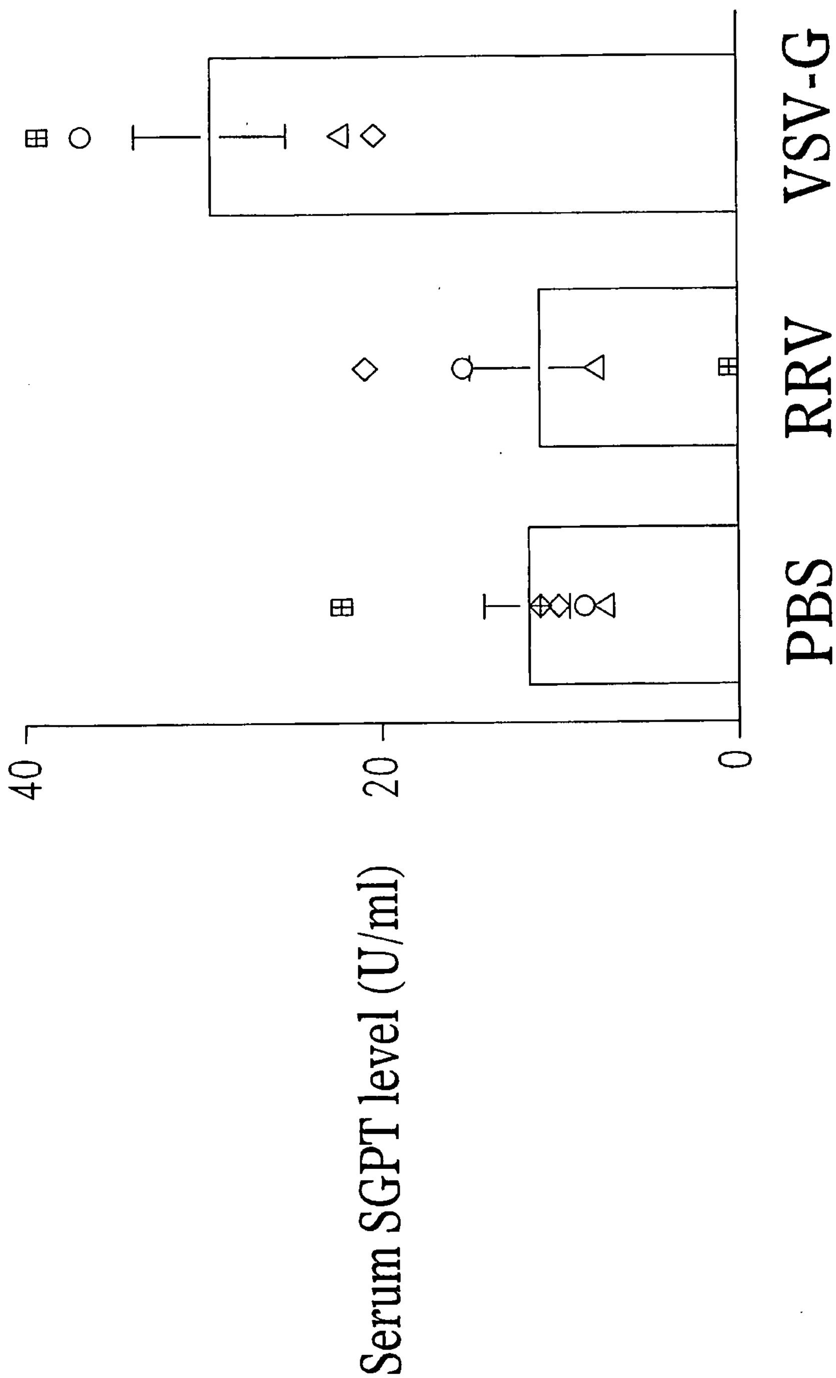

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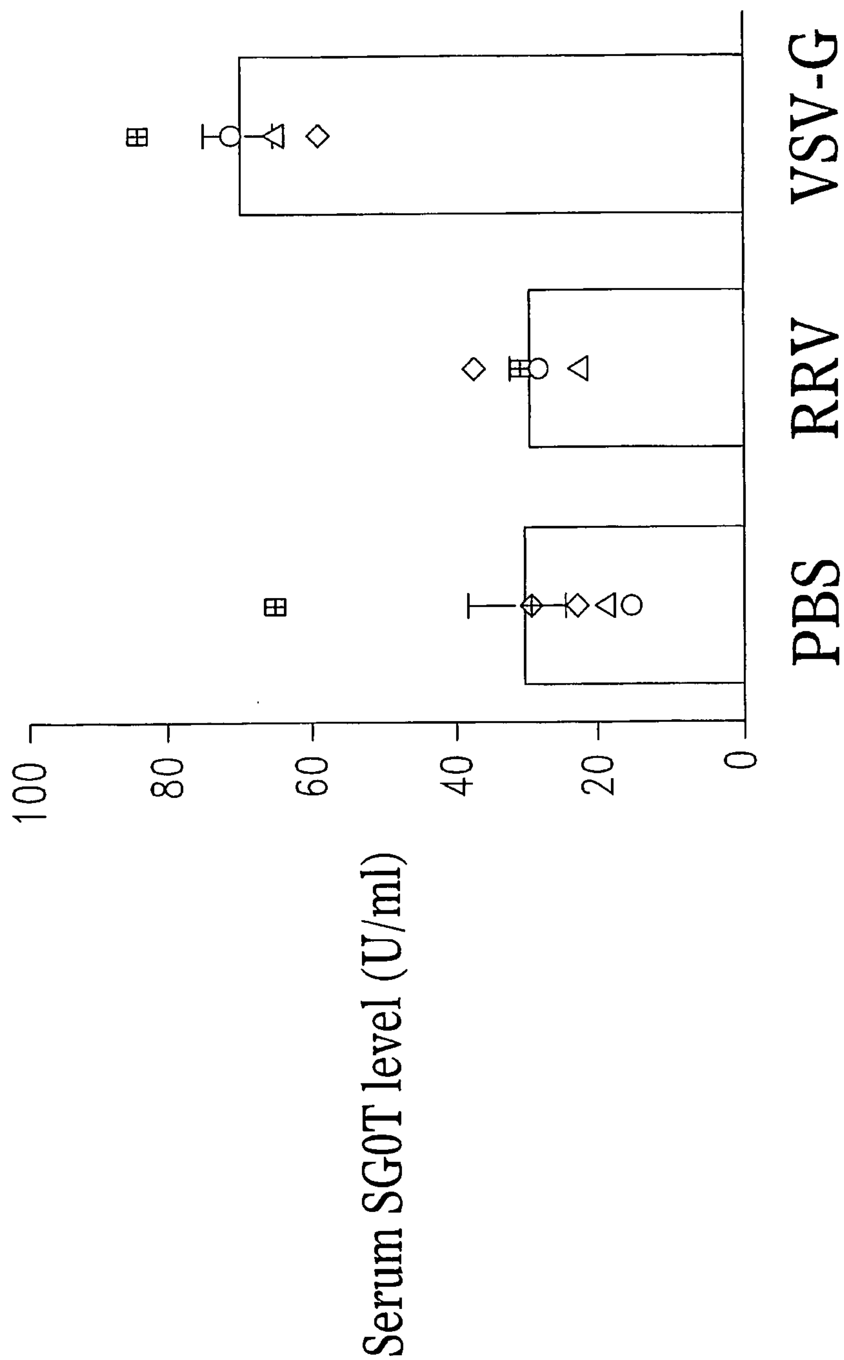

Fig. 2B

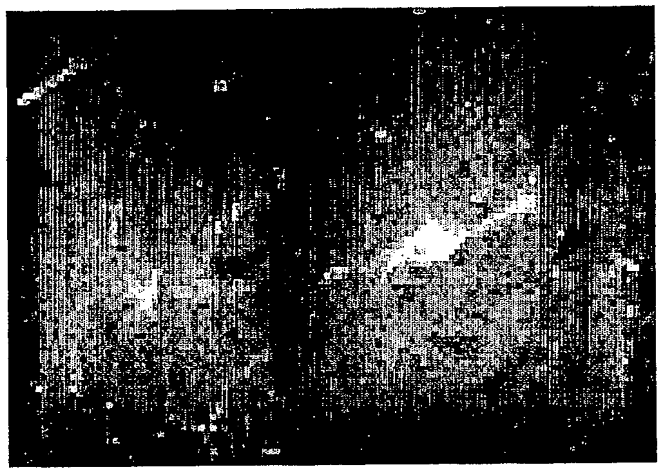

Fig. 3C

Fig. 3B

Fig. 3A

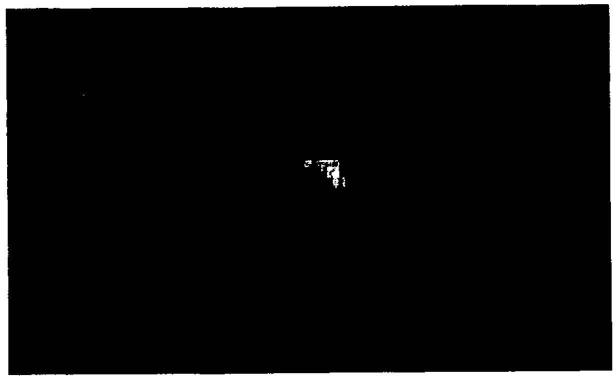

Fig. 4A

Fig. 4B

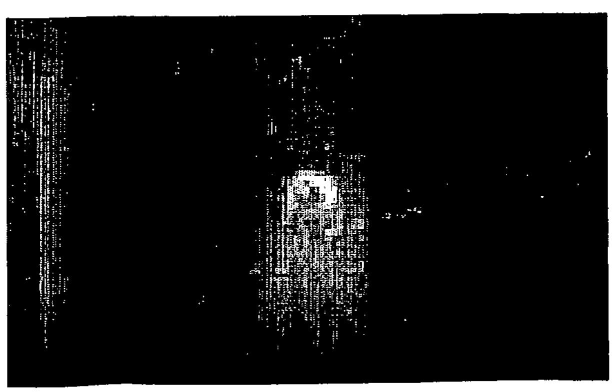

Fig. 4C

Fig. 5A

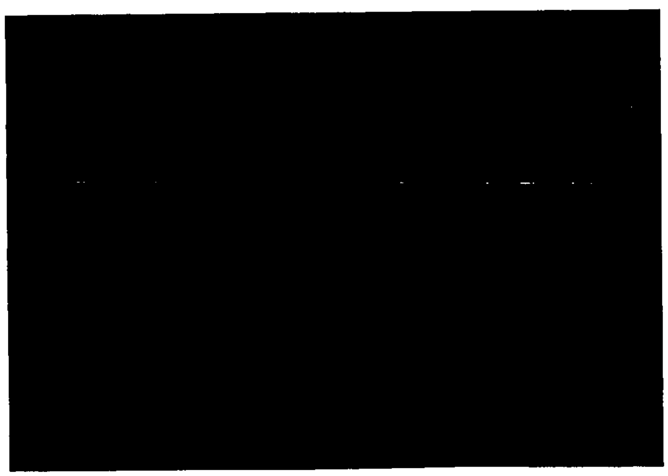

Fig. 5B

Fig. 5C

Cell Type	% Transduced
Neurons	$7.0 \pm 6.5\%$
Astrocytes	$56.5 \pm 17.2\%$
Microglia	$9.9 \pm 5.5\%$
Oligos	26.6%

Fig. 6

Apical

Basolateral

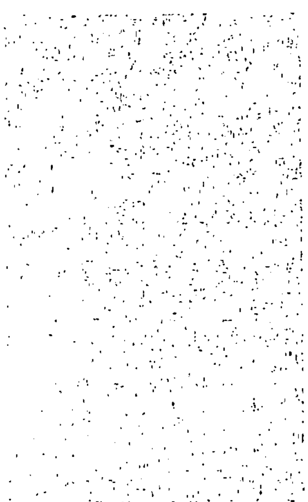

Fig. 7A

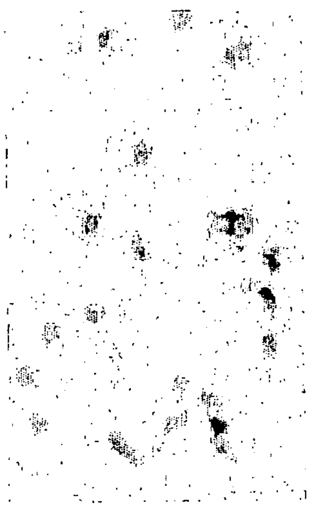

Fig. 7B

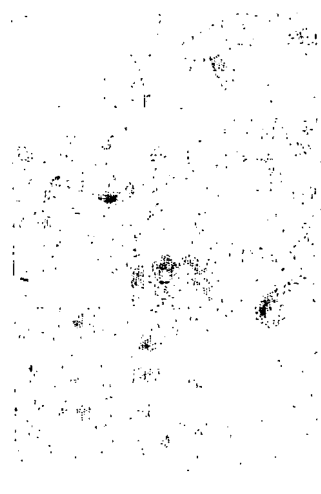

Fig. 7C

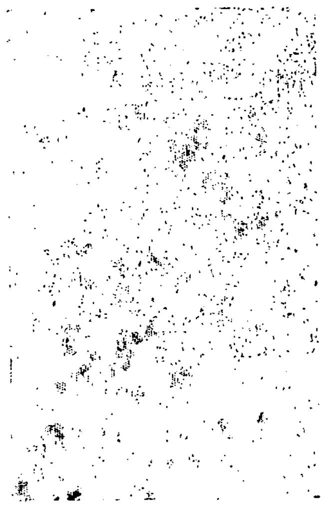

Fig. 7D

	......L I A L S C I C R I F T K Y I G.	(SEQ. ID. NO: 1)
	Transmembrane Domain	
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C673A	......L I A L S C I A R I F T K Y I G.	(SEQ. ID. NO: 3)
C671S	......L I A L S S I C R I F T K Y I G.	(SEQ. ID. NO: 4)
C673S	......L I A L S C I S R I F T K Y I G.	(SEQ. ID. NO: 5)
Y679Stop	......L I A L S C I C R I F T K	(SEQ. ID. NO: 6)
Y676Stop	......L I A L S C I C R K	(SEQ. ID. NO: 7)

Fig. 8

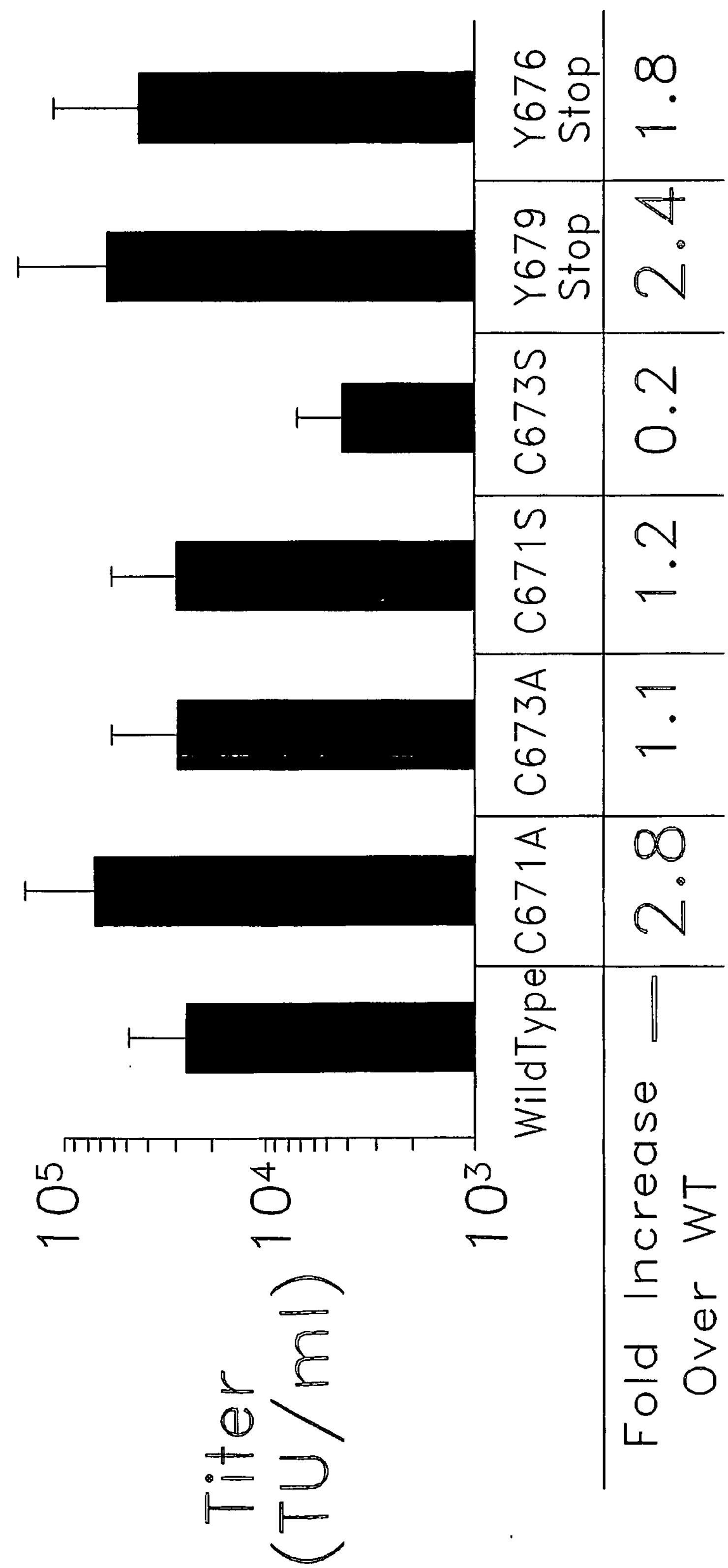
Titer (TU/ml)
10^5
10^4
10^3
WildType
C671A
C673A
C671S
C673S
Y679 Stop
Y676 Stop
Fold Increase Over WT
—
2.8
1.1
1.2
0.2
2.4
1.8

Fig. 9

1	MKTTCFLISL	ILIQGTKNLP	ILEIASNNQP	QNVDSVCSGT	LQKTEDVHLM
51	GFTLSGQKVA	DSPLEASKRW	AFRTGVPPKN	VEYTEGEEAK	TCYNISVTDP
101	SGKSLLLDPP	TNIRDYPKCK	TIHHIQGQNP	HAQGIALHLW	GAFFLYDRIA
151	STTMYRGKVF	TEGNIAAMIV	NKTVHKMIFS	RQGQGYRHMN	LTSTNKYWTS
201	SNGTQTNDTG	CFGALQEYNS	TKNQTCAPSK	IPPPLPTARP	EIKLTSTPTD
251	ATKLNTTDPS	SDDEDLATSG	SGSGEREPHT	TSDAVTKQGL	SSTMPPTPSP
301	QPSTPQQGGN	NTNHSQDAVT	ELDKNNTTAQ	PSMPPHNTTT	ISTNNTSKHN
351	FSTLSAPLQN	TTNDNTQSTI	TENEQTSAPS	ITTLPPTGNP	TTAKSTSSKK
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451	LINAPIDFDP	VPNTKTIFDE	SSSSGASAEE	DQHASPNISL	TLSYFPNINE
501	NTAYSGENEN	DCDAELRIWS	VQEDDLAAGL	SWIPFFGPGI	EGLYTAVLIK
551	NQNNLVCRLR	RLANQTAKSL	ELLLRVTTEE	RTFSLINRHA	IDFLLTRWGG
601	TCKVLGPDCC	IGIEDLSKNI	SEQIDQIKKD	EQKEGTGWGL	GGKWWTSDWG
651	VLTNLGILLL	LSIAVLIALS	CICRIFTKYI	G	

Fig. 10

3220-71692.ST25
SEQUENCE LISTING

<110> McCray, Paul B.
Sanders, David A.
Davidson, Beverly L.

<120> METHODS FOR GENE TRANSFER USING PSEUDOTYPED LENTIVIRUSES

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<150> US 60/356,436
<151> 2001-10-26

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Gly Thr Lys Asn Leu Pro Ile Leu Glu Ile Ala Ser Asn Asn Gln Pro
15                  20                  25                  30

caa aat gtg gat tcg gta tgc tcc gga act ctc cag aag aca gaa gac       147
Gln Asn Val Asp Ser Val Cys Ser Gly Thr Leu Gln Lys Thr Glu Asp
                35                  40                  45

gtc cat ctg atg gga ttc aca ctg agt ggg caa aaa gtt gct gat tcc       195
Val His Leu Met Gly Phe Thr Leu Ser Gly Gln Lys Val Ala Asp Ser
            50                  55                  60

cct ttg gag gca tcc aag cga tgg gct ttc agg aca ggt gta cct ccc       243
Pro Leu Glu Ala Ser Lys Arg Trp Ala Phe Arg Thr Gly Val Pro Pro
        65                  70                  75

aag aat gtt gag tac aca gag ggg gag gaa gcc aaa aca tgc tac aat       291
Lys Asn Val Glu Tyr Thr Glu Gly Glu Glu Ala Lys Thr Cys Tyr Asn
    80                  85                  90

ata agt gta acg gat ccc tct gga aaa tcc ttg ctg tta gat cct cct       339
Ile Ser Val Thr Asp Pro Ser Gly Lys Ser Leu Leu Leu Asp Pro Pro
95                  100                 105                 110

acc aac atc cgt gac tat cct aaa tgc aaa act atc cat cat att caa       387
Thr Asn Ile Arg Asp Tyr Pro Lys Cys Lys Thr Ile His His Ile Gln
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Phe Phe Leu Tyr Asp Arg Ile Ala Ser Thr Thr Met Tyr Arg Gly Lys
        145                 150                 155

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His Lys Met Ile Phe Ser Arg Gln Gly Gln Gly Tyr Arg His Met Asn
175                 180                 185                 190

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        225                 230                 235

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255                 260                 265                 270

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Ser Gly Ser Gly Glu Arg Glu Pro His Thr Thr Ser Asp Ala Val Thr
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        305                 310                 315

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65 70 75 80

Val Glu Tyr Thr Glu Gly Glu Glu Ala Lys Thr Cys Tyr Asn Ile Ser
85 90 95

Val Thr Asp Pro Ser Gly Lys Ser Leu Leu Leu Asp Pro Pro Thr Asn
100 105 110

Ile Arg Asp Tyr Pro Lys Cys Lys Thr Ile His His Ile Gln Gly Gln
115 120 125

Asn Pro His Ala Gln Gly Ile Ala Leu His Leu Trp Gly Ala Phe Phe
130 135 140

Leu Tyr Asp Arg Ile Ala Ser Thr Thr Met Tyr Arg Gly Lys Val Phe
145 150 155 160

Thr Glu Gly Asn Ile Ala Ala Met Ile Val Asn Lys Thr Val His Lys
165 170 175

Met Ile Phe Ser Arg Gln Gly Gln Gly Tyr Arg His Met Asn Leu Thr
180 185 190

Ser Thr Asn Lys Tyr Trp Thr Ser Ser Asn Gly Thr Gln Thr Asn Asp
195 200 205

Thr Gly Cys Phe Gly Ala Leu Gln Glu Tyr Asn Ser Thr Lys Asn Gln

210 215 220

Thr Cys Ala Pro Ser Lys Ile Pro Pro Pro Leu Pro Thr Ala Arg Pro
225 230 235 240

Glu Ile Lys Leu Thr Ser Thr Pro Thr Asp Ala Thr Lys Leu Asn Thr
245 250 255

Thr Asp Pro Ser Ser Asp Asp Glu Asp Leu Ala Thr Ser Gly Ser Gly
260 265 270

Ser Gly Glu Arg Glu Pro His Thr Thr Ser Asp Ala Val Thr Lys Gln
275 280 285

Gly Leu Ser Ser Thr Met Pro Pro Thr Pro Ser Pro Gln Pro Ser Thr
290 295 300

Pro Gln Gln Gly Gly Asn Asn Thr Asn His Ser Gln Asp Ala Val Thr
305 310 315 320

Glu Leu Asp Lys Asn Asn Thr Thr Ala Gln Pro Ser Met Pro Pro His
325 330 335

Asn Thr Thr Thr Ile Ser Thr Asn Asn Thr Ser Lys His Asn Phe Ser
340 345 350

Thr Leu Ser Ala Pro Leu Gln Asn Thr Thr Asn Asp Asn Thr Gln Ser
355 360 365

Thr Ile Thr Glu Asn Glu Gln Thr Ser Ala Pro Ser Ile Thr Thr Leu
370 375 380

Pro Pro Thr Gly Asn Pro Thr Thr Ala Lys Ser Thr Ser Ser Lys Lys
385 390 395 400

Gly Pro Ala Thr Thr Ala Pro Asn Thr Thr Asn Glu His Phe Thr Ser
405 410 415

Pro Pro Pro Thr Pro Ser Ser Thr Ala Gln His Leu Val Tyr Phe Arg
420 425 430

Arg Lys Arg Ser Ile Leu Trp Arg Glu Gly Asp Met Phe Pro Phe Leu
435 440 445

Asp Gly Leu Ile Asn Ala Pro Ile Asp Phe Asp Pro Val Pro Asn Thr
450 455 460

Lys Thr Ile Phe Asp Glu Ser Ser Ser Ser Gly Ala Ser Ala Glu Glu
465 470 475 480

Asp Gln His Ala Ser Pro Asn Ile Ser Leu Thr Leu Ser Tyr Phe Pro

485 490 495

Asn Ile Asn Glu Asn Thr Ala Tyr Ser Gly Glu Asn Glu Asn Asp Cys
500 505 510

Asp Ala Glu Leu Arg Ile Trp Ser Val Gln Glu Asp Asp Leu Ala Ala
515 520 525

Gly Leu Ser Trp Ile Pro Phe Phe Gly Pro Gly Ile Glu Gly Leu Tyr
530 535 540

Thr Ala Val Leu Ile Lys Asn Gln Asn Asn Leu Val Cys Arg Leu Arg
545 550 555 560

Arg Leu Ala Asn Gln Thr Ala Lys Ser Leu Glu Leu Leu Leu Arg Val
565 570 575

Thr Thr Glu Glu Arg Thr Phe Ser Leu Ile Asn Arg His Ala Ile Asp
580 585 590

Phe Leu Leu Thr Arg Trp Gly Gly Thr Cys Lys Val Leu Gly Pro Asp
595 600 605

Cys Cys Ile Gly Ile Glu Asp Leu Ser Lys Asn Ile Ser Glu Gln Ile
610 615 620

Asp Gln Ile Lys Lys Asp Glu Gln Lys Glu Gly Thr Gly Trp Gly Leu
625 630 635 640

Gly Gly Lys Trp Trp Thr Ser Asp Trp Gly Val Leu Thr Asn Leu Gly
645 650 655

Ile Leu Leu Leu Leu Ser Ile Ala Val Leu Ile Ala Leu Ser Cys Ile
660 665 670

Cys Arg Ile Phe Thr Lys Tyr Ile Gly
675 680